Controlling Chronic HV Replication In Vivo With 'Tailor-Made' siRNA-ABC Nanoparticles

Sergio Carmona,[1] Michael Keller,[2] Carol Crowther,[1] Felix H. Salazar,[3,4] Patricia L. Marion,[4] Yukikazu Natori,[5] Patrick Arbuthnot[1*] and Andrew D. Miller[2*]

[1]Hepatitis B Virus Research Unit, Department of Molecular Medicine and Haematology, University of the Witwatersrand Medical School, Private Bag 3, WITS 2050, SOUTH AFRICA.

[2]Imperial College Genetic Therapies Centre, Department of Chemistry, Flowers Building, Armstrong Road, Imperial College London, London SW7 2AY, UK

[3] Stanford University, Stanford CA, USA

[4] Hepadnavirus Testing, Inc. Mountain View CA, USA.

[5]RNAi Co., Cosmos Hongo Bldg. 10F. 4-1-4, Hongo, Bunkyo-ku, Tokyo, 113-0033, JAPAN

*To whom correspondence should be addressed:

E-mail: patrick.arbuthnot@wits.ac.za Tel: +27 (0)11 717 2365 Fax: +27 (0)11 717 2395

E-mail :a.miller@imperial.ac.uk Tel: +44 (0)20 7594 5869 Fax : +44 (0) 20 7594 5803

ABSTRACT

Exploiting RNA interference (RNAi) to silence hepatitis B virus (HBV) gene expression is a new and promising therapeutic approach. Although several studies have shown good HBV knockdown, accomplishing efficient and safe delivery of RNAi effectors in disease models simulating clinical conditions is essential before therapeutic applications can be realized. We have developed a synthetic, 'tailor-made' nanoparticle system (siFECTplus^{TM}) for delivery of antiHBV small interfering RNA (siRNA) sequences *in vivo* with no detectable off-target effects and minimal toxicity and immunological effects. The delivered siRNA is capable of effecting inhibition of markers of HBV replication in cell culture and in a murine hydrodynamic injection model. To assess efficacy in a more stringent model simulating chronic infection in humans, the formulation is administered (siRNA dose 1 mg/kg) every 3 days to HBV transgenic mice over a 4 week period. In these animals, knockdown of hepatitis B S-antigen (HBsAg) secretion is achieved early after administration, but inhibition of markers of viral replication is not sustained. In contrast, viral particle equivalents (VPEs, virions/ml) remain suppressed by 2-3 fold relative to controls even at 28 days, a result that compares favorably with the effects of lamivudine treatment (anti-viral drug; 200mg/kg daily i.p). These observations demonstrate the ability to achieve efficient knockdown of HBV with siFECTplus^{TM}-delivered siRNA under quite stringent conditions.

ABBREVIATIONS:

RNAi: RNA interference; HBV: hepatitis B virus; siRNA: small interfering RNA; HCC: hepatocellular carcinoma; IFN-α: interferon-α; AAVs: adeno-associated viruses; shRNA: small hairpin RNA sequences; shRNAi: small hairpin interfering RNA; pDNA: plasmid DNA; SNALP: stabilized nucleic acid-lipid particle; CDAN: N^4-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; DOPE: dioleoyl-L-α-phosphatidylethanolamine; CPA: cholesteryl-PEG^{350}-aminoxy lipid; PEG^{175}: polyethylene glycol 175; PEG^{350}: polyethylene glycol 350; PEG^{2000}: polyethylene glycol 2000; PEG^{2000}-$(CHO)_2$; polyethylene glycol 2000-dialdehyde; HBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; LDH: lactate dehydrogenase; ALT: alanine transaminase; MHI: murine hydrodynamic injection; HBsAg: hepatitis B S-antigen; HBeAg: hepatitis B E-antigen; OAS-1: Oligoadenylate synthase-1; IFN-β, interferon-β; VPEs: Viral Particle Equivalents.

Approximately 6% of the world's population is chronically infected with hepatitis B virus (HBV) and between 25% and 40% of viral carriers will develop cirrhosis and/or hepatocellular carcinoma (HCC) as complications [1]. Licensed therapies for HBV, which include interferon-α (IFN-α), nucleoside (lamivudine) and nucleotide (adefovir) analogues, are only partially effective and the therapeutic response may not be durable [2-4]. Development of an effective antiviral therapy thus remains an important global priority. Recent demonstrations that the RNAi pathway can be exploited to cause powerful silencing of HBV genes has led to considerable enthusiasm for novel, nucleic acid-based therapies [5, 6]. However, the clinical use of therapeutic anti-viral sequences is dependent on their incorporation into vectors that need to be stable, non-toxic, minimally immunogenic and capable of efficient delivery of sequences to hepatocytes *in vivo*. Although recombinant adenoviruses [7, 8] and adeno-associated viruses (AAVs) [9, 10] have been used successfully to deliver expressed anti-HBV small hairpin RNA (shRNA) sequences to liver cells, these vectors may not be suitable for clinical application given these additional vector requirements.

Current clinical data suggests that virus-based vectors may be highly effective for nucleic acid delivery but suffer from a variety of problems associated with immunogenicity, toxicity and oncogenicity. By contrast, preclinical and current clinical trial data suggest that synthetic non-viral vector systems are much less affected by these problems but are lacking in delivery efficacy. Synthetic vectors also have the inherent flexibility to deliver all varieties of nucleic acids from plasmid DNA (pDNA) to artificial chromosomes, mRNA or small interfering RNAs (siRNAs). Moreover, manufacturing and regulatory issues are also expected to be much less problematic with respect to synthetic vectors. This has lead to the general consensus that virus-based vectors need to be engineered for improved safety profiles and ease of manufacture while synthetic non-viral vector systems should be engineered for improved efficacy [11]. Recently, 'stabilized nucleic acid-lipid particle' (SNALP) non-viral vectors have been described that deliver anti-HBV synthetic siRNAs efficiently to liver in a

murine hydrodynamic injection (MHI) model of transient HBV replication [12]. siRNA delivery was apparently unaffected by the MHI procedure and these vectors have been shown subsequently to be effective for delivery of siRNAs against an endogenous gene in primates[13]. SNALP make for impressive lipid-based vectors but assembly with nucleic acids is neither simple nor flexible and encapsulation efficiency can be variable. In order to advance the therapeutic use of RNAi against HBV, we have since developed an alternative hepatotropic lipid-based siRNA-**ABC** nanoparticle vector system based on the synthetic, self-assembly **ABCD** nanoparticle paradigm for non-viral vector mediated nucleic acid delivery [14]. These novel, simple to assemble and highly flexible formulations comprise siRNA (**A**-component) condensed into **AB** core particles (approx. 70 nm) by means of cationic liposomes (**B**-component), which are then coated by post-coupling with variable amounts of polyethylene glycol (**C**-component) to confer stability with passive targeting to hepatocytes. Here we report on the application of these siRNA-**ABC** nanoparticles for the treatment of HBV in murine models of acute and chronic infection.

RESULTS

siRNA formulations for delivery. Contrary to some expectations, siRNA and pDNA delivery are not equivalent problems [14-16]. Successful siRNA delivery does not have a functional requirement for delivery to cell nuclei and there is no "long term expression" problem, both of which remain serious barriers to properly successful non-viral delivery of pDNA. Hence, it has been noted [14-16] that effective delivery of siRNA *in vivo* should be more straightforward to achieve than effective delivery of pDNA. We were amongst the first to determine that cationic liposome mediated siRNA delivery to cells (siFection) is particularly suitable for siRNA applications, in particular because cationic liposome systems such as siFECTamine® have been specially formulated and adapted for *in vitro* siFection of cells, resulting in gene knockdown efficacy of >90% with minimal toxicity [15]. The cationic liposomes were formulated from the cationic cholesteryl polyamine N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) and the neutral co-lipid dioleoyl-L-α-phosphatidyl ethanolamine (DOPE) in a 45:55 m/m ratio (Fig. 1a).

Given the favorable characteristics of CDAN/DOPE cationic liposomes for siRNA delivery *in vitro*, we have since devised a modular methodology for upgrading the reagent into a form appropriate use *in vivo*. This process involves the stepwise assembly of siRNA-**ABC** nanoparticles from tool kits of chemical components including a variation of the CDAN/DOPE cationic liposome system. The involves the pre-modification of CDAN/DOPE cationic liposomes by the further inclusion of cholesteryl-PEG^{350}-aminoxy lipid (CPA) **1**. CPA contains a short polyethylene glycol 350 (PEG^{350})-moiety equipped with a remote aminoxy functional group, required for the post-coupling of the longer polyethylene glycol **C**-layer (as described below). Post-coupling is essential to provide for optimal flexibility and control over bio-compatible siRNA delivery formulations.

The synthesis of CPA **1** as illustrated (Scheme 1) was completed in two stages. First the synthesis of the protected PEG^{350}-aminoxylinker **2**, and second the solution phase coupling of cholesteryl-amine **3** followed by deprotection. The protected PEG^{350}-aminoxylinker **2**, was synthesized on a 2-chlorotrityl polystyrene resin (Argonaut, USA) using a modular, Fluorenylmethyloxycarbonyl (Fmoc) solid-phase methodology adapted from peptide synthesis. In brief, a short PEG^{175} linker *N*-Fmoc-amido-$dPEG_4^{TM}$-acid **4** (Quanta BioDesign, Inc., USA) was loaded onto resin and converted to amine **5**, after which a second **4** was coupled assisted by a coupling reagent, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Novabiochem, UK). The resulting amine **6** was then capped with *N*-Boc-amino-oxyacetic acid (Novabiochem, UK) affording resin bound **2.** This was in turn cleaved from the resin and coupled to cholesteryl-amine **3** in free solution to yield Boc-protected CPA **7** (71% yield). Cholesteryl-amine **3** was prepared previously from cholesteryl chloroformate **8** (Aldrich, UK) treated with excess ethylene diamine. The formation of CPA **1** was then accomplished by removal of the Boc-group from **7** with 4M HCl in dioxane yielding the CPA **1** (>97% yield by analytical HPLC). This deprotected product was used in subsequent **ABC** nanoparticles formulations and biological studies without the need for further purification.

The core **AB** particles (approx 70 nm in diameter) were initially formulated from siRNA condensed with CDAN/DOPE/CPA (40:50:10, m/m/m) liposomes. These were then stabilized and enabled for passive targeting to hepatocytes by the post-coupling of polyethylene glycol 2000-dialdehyde [PEG^{2000}-$(CHO)_2$] (**C**-layer; 0.1-5.0 mol%) to the surface of the **AB** core particles under aqueous acidic conditions (pH 3.5-4) by means of aminoxy-aldehyde functional group conjugation. The conjugation chemistry results in siRNA-**ABC** nanoparticles (90±20 nm diameter) with oxime coupling linkages (Fig. 1b) that are very robust with respect to degradation at pH 7. Aminoxy-aldehyde functional group conjugation is well known and we have used this chemistry previously [17]. However, the almost unrivalled capacity of these

functional groups to combine as easily in organic solvent as aqueous solution has allowed us to develop a novel, modular and highly flexible way to formulate robust **ABC** nanoparticles and even to include biological ligands to create targeted **ABCD** nanoparticles in due course.

To assess stability of siRNA-**ABC** nanoparticle formulations with respect to aggregation, particle size was determined in the presence of 80% serum over a 4 h time period (Fig. 1c). In the absence of PEG, particle size rapidly climbed above 1000 nm in diameter. However, with increasing mol% incorporations of polyethylene glycol 2000 (PEG^{2000}), increasing particle stability was observed such that the nanoparticles were almost completely stable with respect to aggregation following the inclusion of 5 mol% PEG^{2000}. However, there is a surprisingly high stability conferred by levels of PEG as low as 0.1 mol%.

Vector hepatotropism and experimental toxicology assessment. In order to assess hepatoptropism of the vectors, 4-[^{14}C]-labeled cholesterol (0.5 mol% in place of CDAN) was incorporated into CDAN/DOPE/CPA (40:50:10, m/m/m) liposomes from which siRNA-**ABC** nanoparticles were prepared (as described above) with variable levels of incorporation of PEG^{2000} (0.1-5.0 mol%), and injected via the tail vein of mice. Post injection (1 h), the mice were sacrificed and extracts prepared from various tissues. Labeled cholesterol was found to accumulate preferentially in the liver in all cases (>85% dose, total dose 0.035μCI/mouse) suggesting that the inclusion of PEG was making little difference to the hepatotropism although more aggregation prone particles are much more likely to by sequestered by Kupfer cells (liver phagocytes) than by hepatocytes which are the target cells for HBV treatment (Fig 2a). After this, siRNA labeled with cyanine dye Cy3 (red colour) was also visualized by microscopy in frozen liver sections prepared from mice injected with an Cy3-siRNA-**ABC** nanoparticles with only 1 mol% incorporation of PEG^{2000} (Fig. 2b). Cy3-siRNA was clearly detected in the vicinities of propidium iodide stained hepatocyte nuclei in large aggregate and microaggregate form, but not in other tissues (not shown), thereby confirming specific

delivery of siRNA to the liver. The cellular localization behavior of Cy3-siRNA within hepatocytes *in vivo* is similar to the situation observed in cells *in vitro* post-delivery of fluorescein-labelled siRNA [15]. Potential toxic effects of siRNA-**ABC** nanoparticles (PEG^{2000}; 1 mol%) were investigated experimentally *in vivo* using markers of hepatic, renal and haematological damage. Serum activity of alanine transaminase (ALT; Fig 2c), which is a liver specific indicator of hepatocyte damage, and of lactate dehydrogenase (LDH); Fig 2c), a general marker of cell lysis, were not elevated in the serum of control and siRNA-**ABC** nanoparticles (PEG^{2000}; 1 mol%)-treated animals. Also, renal function was not affected by the vectors as determined by an absence of perturbation in the concentrations of urea, creatinine and electrolytes in the presence of vectors (data not shown). Assessment of stained liver sections and peripheral blood smears analysis from animals that had been treated regularly with siRNA-**ABC** nanoparticles (PEG^{2000}; 1 mol%) for a 4 week period did not show evidence for unintended harmful effects either (data not shown). Collectively these experimental toxicity data indicated that siRNA-**ABC** nanoparticles (PEG^{2000}; 1 mol%) have passive hepatotropic properties but no obvious toxic effects. From this point on, siRNA-**ABC** nanoparticles (PEG^{2000}; 1 mol%) will be referred to as siFECTplus^{TM} nanoparticles for ease of discussion.

Antiviral efficacy of siRNAs in culture and in murine hydrodynamic injection models of viral replication. The initial anti-HBV efficacy of a panel of 7 different siRNAs delivered by siFECTamine was determined in cultured liver-derived Huh7 cells expressing the HBV genome (Table 1). siRNAs were designed to avoid homologous targets found within the human or mouse genomes, and also according to predicted susceptibility of HBV targets to RNAi-mediated silencing [18]. Of the 7 different siRNAs designed, 3 were unable to bring about any HBV protein down-regulation. Of the remaining 4, only siRNA 1410 and siRNA 1794 appeared to mediate reliable HBV protein down-regulation and effective inhibition of

hepatitis B S-antigen (HBsAg) production (>50%) (Fig. 3a). Hence only siRNA 1410 and siRNA 1794 were selected for further studies *in vivo*.

In order to assess HBV gene knockdown *in vivo*, a murine hydrodynamic injection (MHI) model of viral replication was employed initially. Levels of liver delivery of HBV replication competent pDNA were shown to be equivalent between MHI mice as determined by staining for β-galactosidase activity expressed from a pDNA vector that was co-injected with the HBV replication competent pDNA (data not shown). 6 h after high-pressure tail vein injection of HBV replication competent plasmid, siRNA formulations (siRNA dose: 1 mg/kg) were administered intravenously and HBsAg and viral counts were measured thereafter. Compared to controls, injection of siRNA1410 or siRNA1794 within siFECTplus™ nanoparticles effected significant knockdown of HBsAg at time points of 2 and 4 days after intravenous administration (Fig. 3b). This inhibitory effect was confirmed by knockdown of circulating viral particle equivalents at 96 hours after hydrodynamic injection (Fig. 3c).

Antiviral efficacy of siFECT plus-mediated delivery of siRNA in HBV transgenic mice. In order to assess knockdown in a model of HBV replication that more closely resembles the situation in patients who are chronic carriers of HBV, non viral vector formulations were administered to HBV transgenic mice. These animals had been propagated after stable integration of greater than genome length HBV sequences [19]. HBV particles are constitutively produced and the number of circulating viral particle equivalents is in the range from 0.5-1.0×10^7 per ml. siRNAs were administered intravenously every 3 days (each siRNA dose: 1 mg/kg) for a 4 week period. Interestingly, siRNA 1410 delivered within siFECTplus™ nanoparticles initially caused a decrease in HBsAg (up to 15 days), but with time the concentration of this antigen increased to that which was similar to the controls (Fig. 4a). Similarly, at day 28, there was no significant difference in the serum HBeAg concentrations when comparing control mice to those which received siFECTplus™ nanoparticles (Fig. 4b).

In contrast, viral particle equivalents (VPEs, virions/ml) remained suppressed by 2-3 fold relative to controls even at 28 days, a result that compared favorably with the effects of lamivudine (anti-viral drug; 200mg/kg daily i.p.) treatment (Fig. 4c). Similar effects were also seen with cellular HBV S antigen mRNA concentrations (Fig. 4d). These latter data may reflect the fact that these markers are more sensitive indicators of viral replication knockdown than serum HBsAg and hepatitis B E-antigen (HBeAg) concentrations. These data also suggest that our siFECTplus^{TM} nanoparticles may be loosing some capacity with prolonged storage (at 4 $^{\circ}$C) so that the efficacy of encapsulated siRNAs diminishes with time. This issue will need to be investigated further.

Interferon response in vivo. Oligoadenylate synthase-1 (OAS-1) and interferon-β (IFN-β) mRNA concentrations were measured in the livers of treated transgenic mice to determine whether there was evidence for activation of the interferon (IFN) response caused by siRNA administration (Fig 4e). The positive control was a group of mice that had been treated for 6 h with poly I:C, an inducer of the IFN response, using the hydrodynamic injection procedure. Comparisons to controls revealed that both siRNAs administered within siFECTplus^{TM} nanoparticles did not induce activation of the IFN response genes. This supports an interpretation that inhibitory effects on markers of HBV replication were not a result of an unintended toxic immunostimulatory IFN response mechanism that activates apoptosis.

DISCUSSION

One of the most important challenges for development of RNAi-based HBV/HCV therapy is optimization of siRNA delivery to liver hepatocytes. Thus far both viral and non-viral vectors have been used to deliver nucleic acids to the liver *in vivo*, typically by the systemic administration route that is generally preferred to intrahepatic/intraportal injection of vectors. Recent advances have described cholesterol-linked siRNA wherein the siRNA is substantially modified chemically as well on β-D-ribofuranose and in phosphodiester linkages [20]. Otherwise, synthetic, non-viral SNALPs have been described that have been found to deliver siRNA to murine liver for HBV treatment in an MHI model of HBV replication [12], and siRNA to monkey liver for endogenous knock-down of ApoB gene as proof of concept for treatment of hyperlipidemia [13]. The application of SNALP delivery ensured that siRNA chemical modifications could be minimized. In our case, we have extended the range of synthetic, non-viral siRNA delivery to murine liver in order to obtain proof of concept for HBV treatment in a chronic as well as an acute model of HBV infection, and in so doing introduce the reality of modular, self assembly **ABC** nanoparticles capable of repeat administration and delivery of chemically unmodified siRNA to murine liver without apparent toxicity. We contend that the reality of the **ABC** nanoparticles represented here is that their assembly from tool-kits of chemical components offers up a realistic possibility of tailor-made, synthetic delivery **ABC/ABCD** nanoparticle solutions for many other applications beyond liver, on the understanding that the biological barriers to delivery are adequately understood as well as the target cells for the correct resolution of pathology.

Having said this, we would like to note that stability parameters remain to be optimized in the siFECTplus™ nanoparticles described here. We note that the efficacy of the nanoparticle administration was found to decline with time according to some viral markers consistent with a slow loss in nanoparticle function during storage. Increasing PEG^{2000} mol% may well

solve this problem. So too should further "lipid engineering" of the **B**-layer to introduced additional elements of stability without impairing nucleic acid delivery efficacy [21, 22]. Nevertheless, the selected siFECTplus™ nanoparticles still represent a key proof of concept for effective self-assembly **ABC** nanoparticle-mediated repeat dose delivery of siRNA to murine hepatocytes producing pure siRNA-mediated anti-viral effects without any obvious toxicity. In this respect, the lack of even mild liver transaminitis in comparison with the use of SNALP-mediated delivery [13, 14] is interesting and important.

Useful comparisons can also be made with virus-mediated "delivery" of *in situ* shRNAi. To many, the most promising vectors for delivery of expressed RNAi effectors have been considered to be recombinant adenoviruses and AAVs [9]. Both adenoviruses and AAVs are capable of transducing liver cells *in vivo* with high efficiency. Adenoviruses have been used successfully in 2 studies to deliver shRNAi sequences that silence HBV gene expression in transgenic models of HBV replication [7, 8]. Using an indirect approach, adenoviral vectors expressing RNAi effectors against cellular HCV replication cofactors effectively silenced endogenous genes and inhibited markers of HCV replication in Huh7 cells [23]. However, AAV mediated delivery of shRNAi was not without serious toxicity issues, thought to be due to μRNA interference. Moreover, general toxicity of recombinant adenoviruses and an immune response to the viral antigens need to be addressed before clinical applications would become possible. For instance, adenoviruses induce strong innate and adaptive immune responses that may cause toxicity and limit repeated administration [24]. Hence as a result of the work presented here, we are now of the opinion that non-viral siRNA delivery technologies can be matured for clinical use on a timescale competitive with viral vector technology development, and may even become the preferred method for many applications going forward.

METHODS

General Synthesis. Boc-amino-oxyacetic acid and HBTU were obtained from Novabiochem (CN Biosciences, UK). *N*-Fmoc-amido-dPEG$_4$™-acid was purchased from Quanta BioDesign Ltd. (Powell, OH, USA). PS-Carbodiimide and PS-Chlorotrityl-Cl resins were obtained from Argonaut Technologies, Inc. (Foster City, CA, USA). All other chemicals were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated. Dried dichloromethane was distilled with phosphorus pentoxide; other solvents were purchased pre-dried or as required from Sigma-Aldrich (Dorset, UK) or BDH Laboratory Supplies (Poole, UK). HPLC-grade acetonitrile was purchased from Fisher Chemicals (Leicester, UK) and other HPLC-grade solvents from BDH Laboratory Supplies (Poole, UK). Thin layer chromatography (TLC) was performed on pre-coated Merck-Kieselgel 60 F_{254} aluminium backed plated and revealed with ultraviolet light, iodine, acidic ammonium molybdate (IV), acidic ethanolic vanillin, or other agents as appropriate. Flash column chromatography was accomplished on Merck-Kieselgel 60 (230-400 mesh). Mass spectra were recorded using Bruker Esquire 3000, VG-7070B or JEOL SX-102 instruments. ^1H- and ^{13}C- NMR were recorded on Advance Brucker 400 Ultrashield ™ machine using residual isotopic solvent as an internal reference (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, br = broad singlet). Analytical HPLC (Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector) was conducted on a Vydac C4 peptide column with gradient 0.1 % aqueous trifluoroacetic acid (TFA) to 100 % acetonitrile (0.1 % TFA) [0-15 min.], then 100 % acetonitrile (0.1 % TFA) [15-25 min], then 100 % methanol [25-45 min].

Synthesis of **2**. Chlorotrityl Chloride resin (1.27 mmol/g loading, 55 mg, 0.070 mmol) was swollen in dichloromethane for 16 h. The resin was loaded with *N*-Fmoc-amido-dPEG$_4$™-acid **4** (102 mg, 0.209 mmol) and assisted by Hünig base (60 µl, 0.349 mmol) in dimethyl formamide (DMF) (15 ml) for 1 h, followed by capping by acetic anhydride (10% w/v) in

DMF with Hünig base (3 equiv.). Fluorenylmethyloxycarbonyl (Fmoc) removal was achieved with piperidine (20% v/v) in DMF (2×5 min) followed by extensive washing with DMF. The resulting resin-bound free amine **5** was then reacted again with *N*-Fmoc-amido-dPEG$_4$™-acid **4** (102 mg, 0.209 mmol) that was necessarily pre-activated with the coupling reagent 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (132.5 mg, 0.209 mmol) and Hünig base (60 µl, 0.349 mmol) in DMF (15 ml). After 1 h a second capping by acetic anhydride (10 % v/v) was performed in the presence of Hünig base (3 equiv.). Finally, Fmoc was removed once more (as above) yielding resin-bound free amine **6** that was immediately capped by *N*-Boc-amino oxyacetic acid (40 mg, 3 equiv.) with the assistance of HBTU (3 equiv.) and Hünig base (5 equiv.) in DMF (15 ml) to yield the desired resin-bound product **2**. Resin bound **2** was cleaved in a solution consisting of trifluoroethanol 50 % (v/v) in dichloromethane (3 ml) over 4 h to yield a crude residue **2** (40 mg, 0.058 mmol). ∂_H ($CDCl_3$) 1.48 (9H, Boc), 2.51 (2H, t, J = 6.1 Hz, ~C<u>H$_2$</u>CO$_2$H), 2.59 (2H, t, J = 6.05, ~<u>CH$_2$</u>CONHCH$_2$~), 3.45 and 3.52 (2H and 2H, m, CONH<u>CH$_2$</u>CH$_2$), 3.55 – 3.7 (28H, m, <u>CH$_2$</u>OCH$_2$ and CH$_2$O<u>CH$_2$</u>), 3.77 (4H, m, NHCH$_2$<u>CH$_2$</u>O), 4.34 (2H, s, BocHNO<u>CH$_2$</u>CONH), 7.0 (1H, m, BocN<u>H</u>O), 7.9 (1H, m, CH$_2$N<u>H</u>COCH$_2$) and 8.3 (1H, m, CH$_2$N<u>H</u>COCH$_2$). ∂_C ($CDCl_3$) 28.2 (Boc), 35.1 (~C<u>H$_2$</u>CO$_2$H), 36.8 (~<u>CH$_2$</u>CONHCH$_2$~), 38.98 and 39.24 (CONH<u>CH$_2$</u>CH2), 66.7 and 67.3 (<u>CH$_2$</u>CH$_2$CO), 69.6 and 69.9 (NHCH$_2$<u>CH$_2$</u>O), 70.3-70.7 (<u>CH$_2$</u>OCH$_2$ and CH$_2$O<u>CH$_2$</u>), 75.8 (BocHNO<u>CH$_2$</u>CONH), 82.5 (quaternary, Boc), 158 (CO, Boc), 169.3 and 171.8 (quaternary, CH$_2$NH<u>C</u>OCH$_2$) and 173.6 (quaternary, <u>C</u>O$_2$H). ESI-MS 684.30 $(M\text{-}H)^+$.

Synthesis of **3**. Cholesteryl chloroformate **8** (7.5 g, 0.0167 mol) was dissolved in ethylene-1,2-diamine (180 ml) and the mixture stirred for 18 h. The reaction mixture was then quenched with water and extracted with dichloromethane. The organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to afford a residue which was purified by flash column

chromatography [CH_2Cl_2 : MeOH : NH_3 192 : 7 : 1 → CH_2Cl_2 : MeOH : NH_3 92 : 7 : 1 (v/v)] giving the pure cholesteryl-amine **3** (5.5 g, 0.0116 mol, 73%) as a white solid (mp 175-177°C): FTIR (nujol mull) υ_{max} 3338 (amine), 2977 (alkane), 2830 (alkane), 1692 (carbamate) cm^{-1}; ^1H-NMR ($CDCl_3$) δ 0.66 (3 H, s, H-18), 0.838-0.854 (3 H, d, H-27 (J= 6.4 Hz)), 0.842-0.858 (3 H, d, H-26 (J = 6.4 Hz)), 0.890-0.906 (3 H, d, H-21 (J = 6.4 Hz)), 0.922 (3 H, s, H-19), 1.02-1.63 (21 H, m, H-1, H-9, H-11, H-12, H-14, H-15, H-16, H-17, H-20, H-22, H-23, H-24, H-25), 1.76-2.1 (5 H, m, H-2, H-7, H-8), 2.22-2.36 (2 H, m, H-4), 2.79-2.81 (2 H, m, $H_2NC\underline{H_2}$), 3.197-3.210 (2 H, m, $H_2NCH_2C\underline{H_2}$), 4.52 (1 H, m, H-3), 5.31 (1 H, s, H-6); ^{13}C-NMR ($CDCl_3$) δ 11.78 (C-18), 18.64 (C-21), 19.26 (C-19), 20.96 (C-11), 22.49 (C-26), 22.75 (C-27), 23.7 (C-23), 24.20 (C-15), 27.92 (C-25), 28.09 (C-2), 28.16 (C-16), 31.77 (C-8), 31.81 (C-7), 35.72 (C-20), 36.09 (C-22) 36.46 (C-10), 36.91 (C-1), 38.50 (C-24), 39.43 (C-4), 39.64 (C-12), 42.2 (C-13), 41.70 ($H_2NCH_2\underline{C}H_2$), 43.55 ($H_2N\underline{C}H_2$), 49.91 (C-9), 56.04 (C-17), 56.59 (C-14), 74.20 (C-3), 122.39 (C-6), 156.39 (C=O); MS (ESI +ve) 473 (M+H); HRMS (FAB +ve) calcd. for $C_{30}H_{53}N_2O_2$ (M+H) 473.4119 found 473.4107.

Synthesis of **7.** Crude **2** (40 mg, 0.058 mmol) in anhydrous dichloromethane was combined with 4-dimethylaminopyridine (DMAP (22 mg, 0.18 mmol), HBTU (24 mg, 0.063 mmol) and cholesteryl amine **3** (28 mg, 0.06 mmol) and the mixture stirred at ambient temperature under a nitrogen atmosphere for 15 h. The reaction was quenched with 7 % aqueous citric acid and extracted with dichloromethane. The dried ($MgSO_4$) extracts were concentrated *in vacuo* to afford a residue which was purified by flash column chromatography (gradient CH_2Cl_2:MeOH:H_2O) affording pure Boc-aminoxy–PEG^{350}-cholesteryl lipid **7** (47 mg, 0.0411 mmol, 71 %). ^{1}H NMR (400 MHz, $CDCl_3$:MeOD) 5.32 (m, 1H, Chol C6), 4.35 (m, 1H, Chol C-3), 4.28 (s, 2H, (CO)$C\underline{H_2}ONH_2$), 3.67 (4H, m, $NHCH_2\underline{CH_2}O$), 3.56-3.61 (24H, m, $\underline{CH_2}OCH_2$ and $CH_2O\underline{CH_2}$), 3.56 (2H, m, $\underline{CH_2}CH_2CO$), 3.50 (2H, m, $\underline{CH_2}CH_2CO$), 3.35 and 3.43 (2H and 2H, m, $CONHCH_2CH_2$), 3.24 (m, 2H, CholO(CO)$NHC\underline{H_2}CH_2$), 3.18 (m, 2H,

CholO(CO)NHCH$_2$C$\underline{H}_2$), 2.42 (4H, m, ~C$\underline{H}_2$CO$_2$H and ~$\underline{CH}_2$CONHCH$_2$~), 2.27 (m, 2 H, Chol C-24), 1.46 (s, 3 H, Boc), 0.94 –2.10 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 1.0 (s, 3 H, Chol C-19), 0.89 (d, 3 H, J = 6.4, Chol C-21), 0.83, 0.82 (2 x d, 6 H, J = 6.5 and 2.0 Hz), 0.68 (s, 3 H, Chol C-18); ^{13}C NMR (100 MHz, CDCl$_3$) 173.6 (quaternary, $\underline{C}$O$_2$H), 173.3, 172.8 and 170.5 (NH($\underline{C}$O)CH$_2$ONH$_2$), 158.5 (Boc), 156.6 (O$\underline{C}$ONH), 140.166 (C-5), 122.92 (C-6), 82.61 (Boc), 75.77 ((CO)$\underline{C}$H$_2$ONH$_2$), 74.99 (C-3), 70.4-70.8 ($\underline{CH}_2$OCH$_2$ and CH$_2$O$\underline{CH}_2$), 69.81 and 70.04 (NHCH$_2\underline{CH}_2$O), 67.56 and 67.53 ($\underline{CH}_2$CH$_2$CO), 56.7 (C-14), 56.55 (C-17), 50.5 (C-9), 42.7 (C-13), 40.63 and 39.81 (CholO(CO)NH$\underline{CH}_2\underline{CH}_2$) 40.14 (C-4), 39.88 and 39.58 (CONH$\underline{CH}_2$CH$_2$), 39.25 (C-12), 38.94 (C-24), 37.3 (C-1), 36.9 (C-10), 36.95 (~$\underline{CH}_2$CONHCH$_2$~), 36.90 (~C$\underline{H}_2$CO$_2$H), 36.55 (C-22), 36.17 (C-20), 32.28 (C-8), 32.26 (C-7), 28.5 (C-16 and C-2 overlapping), 28.36 (Boc and C-25), 24.6 (C-15), 24.17 (C-23), 22.99 (C-26), 22.73 (C-27), 21.4 (C-11), 19.6 (C-19), 18.96 (C-21) and 12.11 (C-18). ESI-MS 1162.40 [M+K].

Synthesis of **1.** Boc-aminoxy–PEG^{350}-cholesteryl lipid **7** (40 mg, 0.035 mmol) in propan-2-ol (2 ml) was treated with 4 M HCl in dioxane (2 ml) and the mixture stirred at room temperature for 3 h. The solvents were removed *in vacuo* affording CPA **1** (37 mg, 98 %);. ^{1}H NMR (400 MHz, d$_4$-MeOD) 5.31 (m, 1H, Chol C6), 4.57 (s, 2H, (CO)C$\underline{H}_2$ONH$_2$), 4.38 (m, 1H, Chol C-3), 3.69 (4H, m, NHCH$_2\underline{CH}_2$O), 3.53-3.62 (28H, m, $\underline{CH}_2$OCH$_2$ and CH$_2$O$\underline{CH}_2$), 3.37 and 3.43 (2H and 2H, m, CONH$\underline{CH}_2$CH$_2$), 3.26 (m, 2H, CholO(CO)NHC$\underline{H}_2$CH$_2$), 3.19 (m, 2H, CholO(CO)NHCH$_2$C$\underline{H}_2$), 2.45 (4H, m, ~C$\underline{H}_2$CO$_2$H and ~$\underline{CH}_2$CONHCH$_2$~), 2.27 (m, 2 H, Chol C-24), 0.94 –1.99 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 0.97 (s, 3 H, Chol C-19), 0.87 (d, 3 H, J = 6.4, Chol C-21), 0.80, 0.82 (2 x d, 6 H, J = 6.5 and 2.0 Hz), 0.64 (s, 3 H, Chol C-18); ^{13}C NMR (100 MHz, CDCl$_3$) 173.6 (quaternary, $\underline{C}$O$_2$H), 173.3, 172.8 and 170.5 (NH($\underline{C}$O)CH$_2$ONH$_2$), 157.6 (O$\underline{C}$ONH), 140.16 (C-5), 122.94 (C-6), 75.03 (C-3), 71.90 ((CO)$\underline{C}$H$_2$ONH$_2$), 70.4-70.83

($\underline{CH_2}OCH_2$ and $CH_2O\underline{CH_2}$), 69.54 and 70.14 ($NHCH_2\underline{CH_2}O$), 67.62 (2 x $\underline{CH_2}CH_2CO$ overlapping), 57.12 (C-14), 56.56 (C-17), 50.50 (C-9), 42.7 (C-13), 40.54 and 39.91 (CholO(CO)$NH\underline{CH_2CH_2}$) 40.14 (C-4), 39.88 (C-12), 39.38 and 39.65 ($CONH\underline{CH_2}CH_2$), 38.94 (C-24), 37.3 (C-1), 36.95 (C-10), 36.87 (~$\underline{CH_2}CONHCH_2$~), 36.78 (~$C\underline{H_2}CO_2H$), 36.55 (C-22), 36.17 (C-20), 32.28 (C-8), 32.26 (C-7), 28.5 (C-16 and C-2 overlapping), 28.36 (C-25), 24.6 (C-15), 24.17 (C-23), 22.98 (C-26), 22.73 (C-27), 21.42 (C-11), 19.6 (C-19), 18.96 (C-21) and 12.11 (C-18). ESI-MS 1102.50 $[M+K+Na]^+$. Analytical HPLC: 1 peak, RT 31 min.

Cell culture. Huh7 liver cells were cultured in ISE-RPMI complete medium [RPMI-1640 (Gibco BRL, UK)] made up according to the manufacturer's instructions and supplemented as previously described [7]. Fetal calf serum (FCS) was supplemented to 2.5%, and penicillin (50 IU/ml) and streptomycin (50 µg/ml) (Gibco BRL, UK)] were also added. The cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2. 24 h prior to siFection, the Huh7 cells (150,000) were seeded in 2 cm diameter wells and thereafter incubated for 24 h when a confluency of 60-80 % was reached.

Liposome and siRNA formulations. siRNAs were synthesised using 2'-*O*-ACE-RNA phosphoramidites (Dharmacon, CO, USA). Individual strands were combined in equimolar amounts and then subjected to heat denaturation (95 °C for 5 min.) followed by slow cooling to room temperature to form duplexes. Formulation of siRNA-**ABC** nanoparticles was performed as follows. CDAN.3HCl ([MWt 682]; 271 µl, 4 mg/ml in $CHCl_3$), DOPE ([MWt 744]; 140 µl, 10.7 mg/ml in $CHCl_3$) and CPA **1** ([MWt 1040.5]; 100 µl, 4.4 mg/ml in $CHCl_3$) were combined together in a round-bottomed flask (5 ml) (presilanized with nitric acid followed by dimethyldichlorosilane). Organic solvent was evaporated to dryness (rotary evaporator) producing a thin, lipid-film that was rehydrated by addition of milliQ water (18 MΩ) (1ml) and vortex mixing. The multilamellar liposome formulation (pH 3.5-4) was then

vigorously sonicated (30 min) (Sonomatic® water bath, Longford Ultrasonics) to produce very small unilamellar vesicles (SUVs) (<30 nm). An aliquot (250 µl, 3 mg/ml total lipid concentration) was removed and an aqueous solution of siRNA (0.28 mg/ml) was added dropwise with vigorous vortex mixing, until the lipid:siRNA ratio was 12:1 (w/w), followed by the addition of polyethylene glycol 2000-dialdehyde [PEG^{2000}-$(CHO)_2$] (MWt 2000, 10 mg/ml, 0.1-5.0 mol% of total lipid]. The resulting **ABC** nanoparticle mixture was left for 15 min at ambient temperature and then an aliquot of PBS (approx. 500 µl) was added prior to allowing the mixture to equilibrate for a further 16h at ambient temperature. Final siRNA **ABC** nanoparticle sizes were found to be essentially monodisperse and particle size varied from 90±20 nm in diameter (Photon correlation spectroscopy, N4 plus Coulter Electronics). Final siRNA concentrations were adjusted to 0.1 mg/ml for further experimentation.

Transient transfection of Huh7 cells. siFECTamine™ was use to co-deliver either pCH-9/3091 HBV replication competent target plasmid [25], or a vector constitutively expressing enhanced green fluorescent protein (eGFP) [26], together with relevant siRNA sequences. Briefly, to prepare the complexes, nucleic acid (0.25 pmol pDNA and 20 pmol siRNA) (1 mg total) was added to Opti-MEM (100 µl) (Invitrogen, Carlsbad, CA, USA) and in a separate tube siFECTamine™ (12 mg) as added to 100 µl Opti-MEM. The two mixtures were then combined and incubated at room temperature for up to 20 min. Thereafter siFECTamine™-pDNA/siRNA co-complexes were added to the Huh7 culture medium, which was essentially left unchanged, in order to facilitate siRNA delivery (siFection). Controls included mock-treated cells that are not subject to siFection, and negative control cells that were not subject to particle mediated pDNA delivery of pCH-9/3091. siRNA delivery efficiencies of three independent siFection operations were compared for equivalence by visualisation of the expression of eGFP by fluorescence microscopy [26]. HBsAg secretion into the culture

supernatants was measured daily using the Monolisa (ELISA) immunoassay kit (BioRad, CA, USA).

In vivo *assessment of efficacy of anti HBV siRNAs.* The murine hydrodynamic tail vein injection (MHI) method was initially employed to determine the effects of siRNAs on the expression of HBV genes *in vivo*. Experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A saline solution comprising 10 % of the mouse's body mass was injected via the tail vein over 5-10 secs. The saline solution included a combination of 1 μg target DNA (pCH-9/3091) or 10 μg pLTR□β-gal [27] (a control for hepatic DNA delivery, which encodes constitutively active β-galactosidase marker gene). Post-hydrodynamic injection (6 h), siFECTplus™ nanoparticle solution [200 μl] was injected under low pressure via the tail vein. Control mice were treated with naked siRNA solution in which the siRNA was not complexed to non-viral vectors. Blood was collected from the tail vein over a period of 5 days and HBsAg was measured using the electrochemiluminescence assay (ECLIA) from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Animals were sacrificed after 4 days.

Markers of HBV replication in vivo. To measure effects of siFECTplus™ nanoparticle formulations on circulating virion DNA, total DNA was isolated from 50 μl of the serum of mice on days 3 and 5 after hydrodynamic injection and viral particle equivalents determined using qPCR according to previously described methods [7] with EuroHep calibrating standards [28]. Measurement of concentrations of mRNA encoding HBV and IFN response-related genes was also measured according to previously described methods [7]. All real time PCRs were carried out using the Roche Lightcycler V.2. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. Taq readymix with SYBR green

(Sigma, MO, USA) was used to amplify and detect DNA during the reaction. Thermal cycling parameters consisted of a hotstart for 30 sec at 95 °C followed by 50 cycles of 58 °C for 10 sec, 72 °C for 7 sec and then 95 °C for 5 sec. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. Fixed and unfixed frozen liver sections were processed respectively for immunohistochemical HBV core antigen (HBcAg) detection or for β-galactosidase staining [29]. A rabbit polyclonal antibody against HBcAg (Signet Laboratories Inc., MA, USA) and horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) were used to detect the viral antigen in paraffin embedded sections according to standard procedures.

Experimental Toxicity. NMR1 mice were injected with siFECTplus™ nanoparticle solutions into the tail vein as previously described. Four days after the injection, the mice were anaesthetized, and blood samples collected by cardiac puncture before sacrifice. The blood samples were submitted for haematological analysis, urea and electrolyte concentration determination, alanine transaminase (ALT) and lactate dehydrogenase (LDH) activity determination. Assays were performed in the accredited Haematology and Chemical Pathology Department laboratories of the South African National Health Laboratory Services (NHLS) in Johannesburg.

HBV Transgenic mice. HBV transgenic mice with greater than genome length HBV sequence stably integrated into their genomes that constitutively generate HBV particles [19] were used to assess anti viral efficacy of formulations. All procedures were approved by the Animal Care Committee at Stanford University. siFECTplus™ nanoparticle formulations were prepared as described above and injected via the tail vein. Serum HBsAg was measured using a quantitative sandwich ELISA from Abbott Laboratories, and HBeAg was determined using the electrochemiluminescence assay (ECLIA) from Roche Diagnostics (Mannheim,

Germany) according to the manufacturer's instructions. Circulating viral particle equivalents were determined using real time PCR according to procedures described above.

Statistical Analysis. Data are expressed as the mean ± standard error of the mean. Statistical difference was considered significant when $P<0.05$ and was determined according to the Dunnett's multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

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FIGURE LEGENDS

Figure 1. Chemical components and stability of siFECTamine™ and siFECTplus™. **(a)** Schematic illustration of structures of lipid constituents. **(b)** The aminoxy group of CPA **1** allows convenient addition of PEG^{2000}-$(CHO)_2$ to confer biocompatibility and stealth functions. **(c)** Change in particle diameter of siRNA-**ABC** nanoparticles formulations containing varying amounts of PEG (0.1-5.0 mol%), as a function of time in the presence of 80% serum.

Figure 2. Biodistribution and experimental toxicity assessment of siRNA-**ABC** nanoparticle formulations. **(a)** siRNA-**ABC** nanoparticle formulations (0.1-5.0 mol% PEG) containing 4-[^{14}C]-labeled cholesterol were administered intravenously to mice. Post 1h, the animals were sacrificed, and the radioactivity present in heart, lung, blood, liver, spleen and kidney was determined, plus blood in liver (blood/liver). **(b)** Cy3 labeled siRNA incorporated into siFECTplus™ was administered intravenously. Presence of the nucleic acid was detected using microscopy in the liver 4 h after injection. Serum concentrations of ALT **(c)** and LDH **(d)** 4 d post-administration of saline control or various siFECTplus™ formulations to mice.

Figure 3. Effect of siFECTplus™ formulations on markers of HBV replication in cell culture and murine hydrodynamic injection models. **(a)** HBsAg was determined in the cell culture supernatant at times of 24 and 72 h after transfection of the Huh7 liver-derived line with replication competent HBV plasmid and 4 best siRNA sequences 1410, 1755, 1794 and 1795. Remaining 3 sequences were non-functional (data not shown) **(b)** Serum concentrations of HBsAg at 48 and 96 h after administration of siFECTplus™ formulations (siRNA dose: 1 mg/kg) to mice that had been treated using the hydrodynamic injection procedure. **(c)** Circulating viral particle equivalents (VPEs, virions/ml) at 96 h after hydrodynamic injection

of replication competent plasmid followed by administration of naked siRNAs or siFECTplus™ formulations containing equivalent siRNAs.

Figure 4. Markers of viral replication and immunostimulation in HBV transgenic mice. **(a)** HBsAg concentration was determined in mice that were treated every 3 d with saline, siRNA 1410 alone or in siFECTplus™ plus formulation with siRNA1410 (each siRNA dose: 1 mg/kg) over a period of 4 weeks. **(b)** HBeAg concentration measured post 28 d after intravenous administration of saline, siRNA 1410 alone or in siFECTplus™ plus formulation with siRNA1410 per 3 d (each siRNA dose: 1 mg/kg). **(c)** Circulating VPEs post-intravenous administration after 28 d of lamivudine (200 mg/kg, i.p. daily), siRNA 1410 alone, siRNA 1794 alone, siFECTplus™ with siRNA 1410, or siFECTplus™ plus with siRNA 1794 (each siRNA dose: 1 mg/kg). **(d)** Intrahepatic concentration of HBV mRNA isolated from HBV transgenic mice that had been treated as described above over a period of 28 d. Graphical representation to indicate the ratio of HBV surface to housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. **(e)** Intrahepatic concentrations of OAS1 and IFNβ mRNA at 28 d post-administration of saline, siRNA 1410 alone, siRNA 1794 alone, siFECTplus™ with siRNA 1410, or siFECTplus™ plus with siRNA 1794 As positive control, mice were treated with polyI:C at 6 h before sacrifice, hepatic RNA extraction and measurement of OAS1 and IFNβ markers of interferon response induction.

Scheme 1: Reagents and conditions: i) a) *N*-Fmoc-amido-dPEG$_4$™-acid **4** (3 equiv.), Hünig base (5 equiv.) in DMF, 1 h., r.t.: b) 20% (v/v) Piperidine in DMF (2 x 5 min), r.t.; ii) a) *N*-Fmoc-amido-dPEG$_4$™-acid **4** (3 equiv.), HBTU (3 equiv.), Hünig base (5 equiv.) in DMF, 1 h., r.t.: b) 20% (v/v) Piperidine in DMF (2 x 5 min), r.t.; iii) a) *N*-Boc-amino-oxyacetic acid (3 equiv.), HBTU (3 equiv.), Hünig base (5 equiv.) in DMF, 1 h., r.t.; b) 50% (v/v) 1,1,1-trifluoroethanol in dichloromethane, 4 h, r.t.; iv) ethylene diamine (large excess), r.t., 18 h,

73%; v) a) **2**, HBTU (1 equiv.), DMAP (3 equiv.), dichloromethane, r.t., 15 h, 71%; vi) 4M HCl/ dioxane, propan-2-ol, 3 h, 98%.